Programmed cell death in wheat starchy endosperm during kernel development

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Wheat starchy endosperm is fated to die prior to seed maturation. To reveal the death process of wheat starchy endosperm, morphological variations of the nucleus, the breakage of nuclear DNA, the activities of enzymes related to starch synthesis and antioxidation, and grain weight were investigated. Results showed that wheat starchy endosperm death consisted of two main steps: Nucleus degeneration and subsequent denucleated cell development. Nucleus degeneration showed deformation, chromatin condensation, nuclear envelope disruption, and nuclear residue formation from the degenerative nucleus, which were typical features of programmed cell death (PCD), along with starchy endosperm development. Evans blue staining indicated that starchy endosperm cell death occurred asynchronously among the starchy endosperm cells with initiation points randomly distributed in the endosperm tissue. Enzymes related to starch synthesis and anti-oxidation showed high activities and grain weight increased during nucleus degeneration and denucleated cell development stages. These results suggested that the death of wheat starchy endosperm is a special form of PCD.

Key words: Wheat, starchy endosperm cell, programmed cell death, endosperm development, nucleus degeneration, denucleated cell development.

INTRODUCTION

Programmed cell death (PCD) is an integral part of the development of multicellular organisms and is characterized by a genetically determined program that can orchestrate cell death through changes in nuclear morphology and internucleosomal fragmentation of nuclear DNA (Tomei and Cope, 1991, 1994). In plants, PCD plays an important role in maintaining normal plant development including tracheary element cells, aleurone cells, root cap cells, and somatic embryogenesis. Programmed cell death also takes part in plant reproductive development including reproductive primordium abortion, style transmitting tissue, abortive pollen in male sterility lines, death of three of the megaspores in megaspore genesis, and degeneration of anther tapetum and nucellar cells (Greenberg, 1996; Pennell and Lamb, 1997; Becraft and Yi, 2011). Moreover, PCD may participate in plant-environment interactions, such as root cell death in the formation of aerenchyma in response to hypoxia, and leaf cell death in the hypersensitive response to pathogen attack (Greenberg, 1996, 1997; Pennel and Lamb, 1997).
The development of cereal endosperm includes four stages: syncytial, cellularization, growth and differentiation, and maturation. Differentiation of the cereal endosperm results in four distinct cell types: aleurone cells, starchy endosperm cells, transfer cells, and cells of the embryo surrounding region (Olsen et al., 1999; Olsen, 2001, 2004; Brown and Lemmon, 2007). The bulk of the endosperm is composed of starchy endosperm cells and their primary function is the synthesis of starch and storage proteins. Concurrent with the accumulation of these storage metabolites, starchy endosperm cells become a non-living storage tissue at the final stage of starchy endosperm cell maturation.

The pattern and progression of cell death during endosperm development follows that of cellular development and deposition of the storage reserves. The death of starchy endosperm cells is reported to be a PCD process (Young et al., 1997; Young and Gallie, 1999, 2000). The progress of endosperm PCD is accompanied by an increase in nuclease activity and the internucleosomal degradation of nuclear DNA (Young and Gallie, 1999, 2000). Ethylene has been implicated in orchestrating endosperm PCD. The exposure of developing kernels to ethylene results in the premature onset and accelerated execution of PCD whereas inhibition of ethylene biosynthesis delays PCD (Young et al., 1997; Young and Gallie, 1999, 2000).

Endosperm cells are filled with starch during mid to late developmental stages, and are hard to cut into thin sections for cellular morphology observation. Thus there are few reports on the morphological structure associated with cereal PCD. During PCD of starchy endosperm, storage reserves, especially starch, accumulate and kernel weight increases, but no report can be found on whether the starch synthesis enzyme and antioxidant enzyme are associated with cereal PCD.

To investigate the cytological and physiological features of wheat starchy endosperm PCD, we performed a number of experiments during endosperm development. Starchy endosperm nucleus was observed with transmission electron microscopy (TEM). A variety of nuclear morphology was observed after staining with 4', 6-Diamidino-2-phenylindole (DAPI) using a fluorescent microscope. The breakage of DNA was detected with agarose gels and terminal deoxynucleotidyl transferase (TdT) mediated dUTP nick end labeling (TUNEL). The activities of enzymes which are related to antioxidant and starch synthesis were assayed. Grain weight was also determined during endosperm development.

**MATERIALS AND METHODS**

**Plant materials**

Wheat (*Triticum aestivum L.*) cv. Yangmai 12 was obtained from Agricultural College of Yangzhou University and grown in the experimental field of Yangzhou University, Yangzhou, China during growing seasons. Heads were tagged at flowering. For enzyme assays, 20 kernels were collected from each of three separate ears at the interval of 3 days from 9 days after flowering (DAF). These samples were separated into endosperm and embryo components, frozen in liquid nitrogen and stored at -80°C until analysis.

**TEM observation**

Developing caryopses (6 to 30 DAF in two-day intervals) were harvested for TEM specimen preparation. The endosperms were immediately separated from the caryopses and cut into < 1 mm³ blocks using a razor blade, then fixed in a fixative containing 2.5% glutaraldehyde and 4% paraformaldehyde in 100 mM sodium phosphate buffer (pH 7.2) for 2 h at room temperature followed by 4°C overnight. After three 20 min washes with the same buffer solution, the blocks were postfixed in 1% osmium tetroxide (buffered in 100 mM sodium phosphate buffer, pH 7.2 for 2 h at room temperature, dehydrated in series concentrations of acetone embedded in Spurr resin. The tissue was cut into approximately 70 nm ultrathin section using a diamond knife on an ultramicrotome (Leica EM UC-6, Germany), and then double stained with uranyl acetate and lead citrate. The cellular structures of endosperms were observed and photographed with a Philips Tecnai 12 TEM at an accelerating voltage of 100 kV.

**Viability staining**

For viability staining, median longitudinal and cross sectioned fresh developing kernels (12 to 32 DAF in four-day intervals) were incubated into 0.1% (v/v) aqueous Evans blue solution for 2 min. Stained tissues were rinsed with water for 60 min and photographed using an Olympus SZX7 Zoom Stereo Microscope.

**DNA extraction and fragmentation analysis**

DNA was extracted as previously described by Young and Gallie (1999) with some modification. Dissected endosperms from 20 kernels at the interval of 5 days from 10 DAF were frozen in liquid N₂ and quickly ground into fine powder. Eight ml extraction buffer (100 mM Tris-HCl pH 9.0, 20 mM EDTA, 200 mM NaCl, 1% (w/v) sarcosyl, 1% (v/v) 2-mercaptoethanol) was added and mixed thoroughly with the powder followed by 8 ml phenol/chloroform mixture (1:1). The samples were centrifuged at 8 000 g for 10 min at 4°C. The supernatant was carefully removed from individual samples, ensuring that the pellet was not disturbed. Sodium acetate (pH5.2) and isopropanol (1:3) were added and the samples were left at -20°C for 30 min, after which they were centrifuged at 12 000g for 15 min at room temperature, and washed with 70% (v/v) ethanol. DNAs were suspended in TE buffer (10 mM Tris-HCl pH 8.0, 5 mM EDTA) and DNase free RNase was added to a final concentration of 100 μg/ml. The reaction solution was incubated for 3 h at 37°C. After extracting with phenol/chloroform (1:1), the DNAs were then precipitated with isopropanol and resuspended in TE buffer. The DNAs were resolved on 1.5% (w/v) agarose gels and stained with ethidium bromide.

**Steedman's wax embedding**

Developing kernels at the interval of 5 days from 15 DAF were fixed in 2.0% glutaraldehyde in 100 mM sodium phosphate buffer (pH 7.2) for 2 h at room temperature and then overnight at 4°C. Samples were rinsed with the fixative solution again and dehydrated in series concentrations of ethanol solution. Tissues were embedded in 37°C Steedman's wax which was prepared from PEG 400 distearate and 1-hexadecanol (9:1) as described by
Baluška et al. (1992) and left to polymerize at room temperature. Waxed kernel tissues were cut into approximately 8 μm thick sections on a rotary microtome (Leica RM2145), mounted on slides coated with glycerol albumin, and then dewaxed in absolute ethanol.

**RESULTS**

**Structural changes in starchy endosperm nucleus**

The ultrastructural features of starchy endosperm cell nucleus at growth and differentiation stage are shown in Figure 1. At the early stage of endosperm growth and differentiation, the endosperm cell showed a round-shaped central nucleus with a clear nuclear envelope and one or many nucleoli, an electron-dense cytoplasm enriched with amyloplasts, mitochondria, endoplasmic reticula (ER), and a large quantity of big vacuoles close to the cell wall (Figures 1A and B). At the mid stage of endosperm growth and differentiation, some degenerative features appeared. There were some invaginations visible on the nuclear envelope of the starchy endosperm nucleus, around which mitochondria and ER were also observed (Figure 1C). Some parts of the nuclear envelope protruded toward the space between starch granules (Figure 1D and E). With endosperm development, chromatin gradually became mildly condensed. The condensed chromatin aggregated into clumps and distributed evenly in the nucleus, and the deformation of the nucleus became more apparent (Figure 1D to F). At the early phase of degeneration, most of the nuclear envelopes still maintained the bilayer structure, but in some regions either the inner or the outer nuclear membrane was destroyed, forming transparent vesicles (Figure 1G and H). At the late stage of endosperm growth and differentiation, the endosperm cells were full of starch granules. It was difficult to find the nucleus in the endosperm cells though nuclear residues could still be seen occasionally among starch granules (Figure 1l).

Nuclei with smooth and clear boundaries were observed at the early stage of endosperm development (Figure 2A). The nuclei became deformed and then degenerated with the endosperm development (Figure 2B and C). Some nuclear residues were observed in starchy endosperm cells approaching maturity (Figure 2D).

**DNA fragmentation of starchy endosperm nucleus**

A TUNEL-positive character, which demonstrates the presence of DNA fragmentation, is recognized as one feature of PCD. During wheat starchy endosperm PCD, the deformed nucleus became TUNEL-positive, indicating that DNA breakage occurred (Figure 3). DNA ladder was first detected at 15 DAF. Degradation product amount increased during subsequent seed development as did their shift to species of smaller molecular weight (Figure 4).

**Cell death of starch endosperm cell**

Viability staining provides a means to follow the pattern and progression of cell death during endosperm development. Evans blue stained only those cells which were no longer capable of excluding the dye, indicating a loss of membrane integrity and consequently viability. Because of the asymmetry of the wheat seed, it was

**Antioxidant enzyme assay**

The developing endosperms were homogenized in ice-cold phosphate-buffered saline (PBS) (50 mM, pH 7.0) containing 1% polyvinylpyrrolidone (PVP), and centrifuged at 10 000 g for 10 min at 4°C. The supernatant was used immediately to determine the activities of superoxide dismutase (SOD), peroxidase (POD) and catalase (CAT) as described previously by Rao et al. (1996). The enzyme assay was repeated three times.

**Starch synthesis enzyme assay**

Frozen grains were weighed and homogenized with a pestle in an ice-cold motor, which contained 10 ml of 50 mM HEPES-NaOH (pH 7.5), 10 mM MgCl₂, 2 mM EDTA, 50 mM 2-mercaptopoethanol, 12.5% (v/v) glycerol, and 5% (w/v) insoluble PVP (polyvinylpyrrolidone-40). The preparation procedure of enzyme extraction was similar to that described by Nakamura et al. (1989). The assays of ADP-glucose pyrophosphorylase (AGPP), soluble starch synthase (SSS), granule-bound starch synthase (GBSS), and starch branching enzyme (SBE) activities were carried out as described by Jiang et al. (2004). Enzyme assay was repeated three times.
Figure 1. The ultrastructural morphological changes of nucleus in wheat starchy endosperm cell. (A) 8 DAF, showing the normal nucleus and organelle; (B) 12 DAF, showing the distribution of amyloplasts, protein bodies and vacuoles around the normal nucleus; (C) 14 DAF, showing the slight invagination of nuclear envelope; (D) 18 DAF, showing the deformed nucleus; (E) the magnification of the white square in figure 1D, showing the deformed nucleus among amyloplasts; (F) the magnification of the black square in figure 1D, showing the invagination of nucleus; (G) 20 DAF, showing the deformed nucleus; (H) the magnification of the square in Figure 1G, showing the membrane vesicle structure derived from the degenerative nuclear envelope (arrow); (I) 22 DAF, showing the nucleus residues among the starch granules. N, nucleus; P, protein body; S, starch granule; V, vacuole. Bars, 10 μm (A-D and I) and 1 μm (E-H).

Figure 2. DAPI staining of nucleus in wheat endosperm. (A) 15 DAF; (B) 20 DAF; (C) 25 DAF; (D) 30 DAF. AC, aleurone cell; P, pericarp; SEC, starchy endosperm cell. Bars, 100 μm.

necessary to analyze both longitudinal and cross sections of a developing seed. During early seed development, no staining was observed in the starchy endosperm. Staining within the starchy endosperm was first detected in scattered cells after 12 DAF and the number of stained cells increased substantially with the endosperm development (Figure 5). Staining during endosperm development was not spatially localized in either the longitudinal or cross sections, suggesting that no region or specific endosperm cell type functioned as the site of
Figure 3. DNA breakage by TUNEL labeling in wheat starchy endosperm (25 DAF). (A) DAPI staining; (B) TUNEL labeling; (C) Merged picture of (A) and (B); (D) Merged picture of (A) and phase picture. S, Starch granule. Bars, 20 μm.

Figure 4. DNA electrophoresis analysis oligonucleosomal fragments in wheat endosperm during development. M: DNA marker; Line 1-line 7: endosperm at 10, 15, 20, 25, 30, 35 DAF and maturity, respectively.

initiation for the PCD program, but rather, cell death initiated stochastically throughout the endosperm (Figure 5). At 32 DAF, the entire endosperm was stained (Figure 5). No staining within the embryo or aleurone was observed at any stage of development (Figure 5).

Enzymes and grain weigh changes during endosperm development

The activities of SOD, POD, and CAT in wheat endosperm from 9 to 35 DAF were analyzed (Figure 6). Their activity increased from 9 to 12 DAF. As development progressed, the activity descended after 12 DAF. The rate of decline in SOD activity was much slower, while POD and CAT activity decreased rapidly. The activities of starch synthesis enzymes, AGPP, SSS, GBSS, SBE, during wheat endosperm development are shown in Figure 7. AGPP activities were very low at 9 DAF, increased significantly from 9 to 15 DAF, maintained very high levels from 15 to 30 DAF and decreased at maturity. SSS activities showed a typical “Λ” pattern during endosperm development, with the highest activity at 21 DAF. The GBSS activity pattern was similar to SSS, but the highest activity was found at 18 DAF. SBE activity gradually decreased after 15 DAF, but the rate of decrease was slow. Grain weight increased during endosperm development (Figure 8), which indicated that endosperm cell could synthesize and accumulate storage metabolite during nucleus degeneration and denucleated cell development stages.

DISCUSSION

PCD is characterized by some features, such as nucleus morphological variation, DNA ladder, and TUNEL-positive labeling. Young and Gallie (1999, 2000) reported that the death of starchy endosperm cells in wheat and maize was a PCD process. During early and middle stage, DNA was degraded into 300 to 50 kb DNA fragments. During later stages, the organized degradation of the genome resulted in the appearance of internucleosomal DNA fragments in multiples of 180 bp. Increases in nuclease activities had also been observed during grain development (Young et al., 1997; Young and Gallie, 1999, 2000). The increase in nuclease activities during late endosperm development was accompanied by a reduction in both DNA and RNA content, specifically in the endosperm (Young et al., 1997; Young and Gallie, 1999, 2000). In this paper, the nucleus morphology variation and TUNEL result both indicated that wheat starchy endosperm underwent PCD during its development.

After nucleus degeneration, starchy endosperm still showed high metabolic activity. Enzymes related to starch synthesis showed high activities, and grain weight also increased even with the disappearance of the nucleus. This phenomenon was also reported during rice starchy endosperm PCD (Lan et al., 2004), and was different from common PCD in which the nucleus was the last organelle to disappear during PCD in most animal and plant cells, with cell death immediately after the disappearance of nucleus (Greenberg, 1997; Mittler and Lam, 1995). Reactive oxygen intermediates (ROI) had been implicated in the induction and execution of PCD in plants (Breusegem and Dat, 2006). SOD, POD and CAT were key antioxidant enzymes present in prokaryotic and eukaryotic cells as a first line of defense against the accumulation of ROI (Corpas et al., 2006). After nucleus
Figure 5. Viability staining of cross sections (a–f) and longitudinal sections (A–F) of developing wheat seed. Samples were collected at 12, 16, 20, 24, 28 and 32 DAF, respectively, and assayed for viability by staining with Evans blue. The seeds shown are representative of 10 individual stained seed. Cell death is indicated by the cells stained blue. Bars, 0.5 mm (a–f) and 0.25 mm (A–F).

Figure 6. Dynamic changes of activities of antioxidant enzymes during wheat endosperm development. (A) SOD: U g⁻¹ FW min⁻¹; (B) POD: ΔOD470 g⁻¹ FW min⁻¹; (C) CAT: ΔOD240 g⁻¹ FW min⁻¹. Error bars represent ±S.D. (n=3).
Figure 7. Dynamic changes of activities of starch synthesized relative enzymes during wheat endosperm development. (A) AGPP: nmol (grain min)$^{-1} \times 10^{-3}$; (B) SSS: nmol (grain min)$^{-1} \times 10^{-3}$; (C) GBSS: nmol (grain min)$^{-1} \times 10^{-3}$; (D) SBE: U (grain min)$^{-1} \times 10^{-3}$. Error bars represent ±S.D. (n=3).

Figure 8. Dynamic changes of grain weight during wheat endosperm development. M: maturity. Error bars represent ±S.D. (n=3).

degeneration in wheat starchy endosperm cells, the activities of antioxidant enzymes, especially SOD, stayed high.

Two types of plant PCD have been reported in the literature so far: the first one was similar to animal PCD, in which the degraded products of the dead cell were absorbed by other living cells, for example, PCD of nucellar cells and tapetum cells (Bell, 1996; Dellaporta and Calderon-Urrea, 1994). In the second PCD type, the dead cell itself reused the degraded materials, such as building the cell wall in tracheary elements (Groover and Jones, 1999). The PCD of wheat starchy endosperm was apparently different from the two above-mentioned types. Starchy endosperm cells constituted the bulk of the cereal endosperm and represented the major storage site for starch and protein (Becraft, 2001). PCD could be considered as the last stage of cell differentiation (Wyllie et al., 1984). It may be concluded that wheat starchy endosperm nucleus disintegration, which is the dekaryotization or denucleation of the starchy endosperm
cells, could be seen as a unique pathway that leads to the specialization of cellular functions. The disintegration of the nucleus could make room for accumulating more reserves in the starchy endosperm cell. Dekaryotized or denucleated starchy endosperm cells were specialized to synthesize and accumulate starch and storage proteins, so that their ability to synthesize and accumulate reserves was strengthened during grain filling.

In conclusion, wheat starchy endosperm death consisted of two main steps: nucleus degeneration and subsequent denucleated cell development. Nucleus degeneration showed deformation, chromatin condensation, nuclear envelope disruption, and nuclear residue formation from the degeneration nucleus, which were typical features of PCD. DNA ladder was detected at the middle and late stage of starchy endosperm development. These characters of nucleus degeneration and DNA fragmentation indicated that wheat starchy endosperm cells underwent PCD during development. During nucleus degeneration and denucleated cell development, enzymes related to starch synthesis and antioxidantization showed high activities, and grain weight increased until endosperm cells were completely filled with reserves, which was similar to other cereal starchy endosperm PCD, but different from non-endosperm PCD reported in plants. Therefore, we concluded that the death of cereal starchy endosperm is a special form of PCD in plant.

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